Cell Transplantation, Vol. 17, pp. 587–598, 2008
Printed in the USA.

0963-6897/08 $90.00 + .00
E-ISSN 1555-3892
www.cognizantcommunication.com

Cobalt Protoporpyhrin Reduces Caspase-3,-7 Enzyme Activity in Neonatal Porcine Islets, But Does Not Inhibit Cell Death Induced by TNF-α

Erika Bosio,*† Michela Seveso,* Arben Dedja,*† Giovanni Luca,‡ Mario Calvitti,‡ Riccardo Calafiore,‡ Paolo Rigotti,*† Roberto Busetto,§ Ermanno Ancona,*†#¶ and Emanuele Cozzi*†¶#

*CORIT (Consorzio per la Ricerca sul Trapianto d'Organi), Padua, Italy
†Department of Medical and Surgical Sciences, University of Padua, Padua, Italy
‡Department of Internal Medicine, Section of Internal Medicine and Endocrine and Metabolic Sciences, University of Perugia, Perugia, Italy
§Department of Clinical Veterinary Science, University of Padua, Legnaro, Italy
¶Direzione Sanitaria, Padua General Hospital, Padua, Italy
#Clinica Chirurgica III, Padua General Hospital, Padua, Italy

Apoptotic phenomena observed in vitro following isolation and following transplantation contribute significantly to islet graft loss. Strategies to reduce apoptosis of islet tissue prior to and posttransplantation may improve graft survival and function and reduce the amount of tissue necessary to achieve insulin independence. The expression of cytoprotective proteins is one such strategy that may prolong islet survival. In this light, heme-oxygenase 1 (HO-1) upregulation has been studied in both allo- and xenotransplantation models. In this study, the effect of HO-1 on apoptosis in neonatal porcine islet-like cell clusters (NPICC) was assessed. In in vitro assessments of NPICC apoptosis, NPICC showed a high sensitivity to apoptotic stimulation using a combination of TNF-α and cycloheximide. Stimulation with TNF-α alone was sufficient to induce reproducible apoptotic responses as demonstrated by caspase-3,-7 activation and subdiploid DNA analysis. Dose-dependent, high-level HO-1 protein expression was achieved following culture of NPICC in medium containing either cobalt protoporphyrin (CoPP) or cobalt mesoporphyrin (CoMP). CoPP treatment resulted in the reduction of caspase-3,-7 enzyme activity following TNF-α stimulation. However, such an effect was not associated with a reduction in the levels of cell death. Indeed, the inhibition of caspase enzyme activity resulted in decreased PARP-1 cleavage, which may lead to heightened levels of necrosis in treated NPICC cultures, possibly explaining the observed commitment of NPICC to the death pathway.

Key words: Islet; Porcine; Apoptosis; Necrosis; Metalloporphyrin

INTRODUCTION

Islet transplantation as a treatment and potential cure for type 1 diabetes is a clinical reality (51,54). However, for a number of reasons, it is still not possible to provide such a transplant for all diabetic patients who could benefit from the procedure. While at the forefront is the risk–benefit consideration of insulin therapy versus chronic immunosuppression, a further obstacle to islet transplantation is a severe shortage of tissue suitable for transplantation. Islet transplants often require tissue from more than one donor in order to generate the required tissue mass for a functional graft. Therefore, for the potential number of diabetics who could benefit from such treatment, the organ shortage situation is dire.Successful islet transplantation requires a large amount of donor tissue for reasons related to the cellular nature of the graft. Unlike solid organ grafts, the generation of islets for transplantation requires extensive processing of the pancreas. The isolation process, although in continuous improvement, is inefficient, recovering approximately 20–50% of the islets present in a pancreas (39). In addition, a large number of the islets are lost following postisolation culture and transplantation, in large part due to the induction of apoptosis, mediated by anoikis (11,44), hypoxia (59), activation of the instant blood-mediated inflammatory reaction (IBMIR) (4), and necrosis.

Considering approximately 500,000 people are affected by type 1 diabetes in the US alone (15), and predictions indicating a 3% rise per year in the incidence

Received March 9, 2007; final acceptance December 4, 2007.
Address correspondence to Erika Bosio, Ph.D., Laboratori CORIT, Via dell'Università, 10, Legnaro (PD), 35020 Italy. Tel: +39 049 808 4364; Fax: +39 049 808 4361; E-mail: erika.bosio@unipd.it

of type 1 diabetes globally (42), the demand for islet tissue will always exceed the number of donors available. In this light, approaches to address the tissue shortage include research into alternative tissue sources—for example porcine islets (7,9,27,31,32,38)—and the development of strategies to reduce islet susceptibility to stress and damage induced by isolation and transplantation.

Reducing postisolation stress and apoptosis by improving in vitro islet culture methodologies to enhance islet viability and survival is therefore an important goal. Numerous studies in diverse experimental models have applied a variety of molecules in attempts to reduce the occurrence of apoptotic phenomena both in vitro and in vivo. These studies have included molecules such as Bcl-2 (16,22), erythropoietin (21), caspase inhibitors (23), A20 (24), carbon monoxide (CO) (25), glucagon-like peptide-1 (GLP-1) (29,55), vitamin D_3 (48), and heme-oxygenase-1 (HO-1) (36,45,46,49,56,57) with varying degrees of success. Most recently, very encouraging effects on hypoxia/reperfusion injury and apoptosis in islets have been demonstrated using XIAP (X-linked inhibitor of apoptosis protein) (20). However, to date, none of these approaches have completely eliminated the impact of apoptotic phenomena on the ultimate tissue mass alive and available to control diabetes.

The promising results obtained in islet allotransplantation in rodents by Pileggi and colleagues, using metal porphyrins to upregulate HO-1 (46,47), encouraged us to assess the efficacy of cobalt protoporphyrin (CoPP) as a cytoprotective agent on neonatal porcine islet-like cell clusters (NPICC) in vitro, with the view of developing an in vivo protocol for application in primates. Indeed, the properties of HO-1 make it a particularly attractive molecule with regards to apoptosis intervention (43). The enzyme is one of a family of proteins responsible for the degradation of heme, resulting in the production of carbon monoxide, biliverdin, and free iron. Each of these products has demonstrated protective functions in a number of different models [for review see (43)].

The results presented in this study demonstrate a high sensitivity of NPICC to apoptosis. Following treatment with TNF-α alone, CoPP resulted in the reduction of caspase-3,-7 enzyme activation. However, despite these observations, NPICC death was not reduced in vitro. Indeed, the inhibition of caspase-3,-7 activation by CoPP possibly results in increased levels of NPICC necrosis in a manner similar to that observed following specific inhibition of caspase-3 and -7 by Ac-DEVD-CHO.

MATERIALS AND METHODS

Reagents

Human or porcine TNF-α (Biosource, Nivelles, Belgium) was used at 50 or 100 ng/ml as indicated. Cobalt protoporphyrin IX chloride (CoPP) and cobalt mesoporphyrin chloride (CoMP) (kindly donated by Frontier Scientific Inc.) were assessed at concentrations from 10 to 200 μM. As CoPP at the highest concentration tested (200 μM) resulted in high-level HO-1 expression, without toxic effects on NPICC, this concentration was applied in all subsequent experiments assessing effects on apoptosis. The specific caspase-3,-7 inhibitor, Ac-DEVD-CHO (Promega, Madison, WI, USA), was added to islet cultures 1 h prior to apoptotic stimulation at either 1 or 50 μM. Cycloheximide (CHX) (Sigma, Steinheim, Germany) was prepared as a 10 mg/ml stock solution in 100% ethanol, filter sterilized, and stored at −20°C.

Neonatal Porcine Islet Isolation

White Landrace neonatal piglets (3–5 days old) were anaesthetized by subcutaneous injection of xylazine (3 mg/kg) and Zoletil (7 mg/kg) followed by the IV administration of Contramal (4 mg/kg) and Propofol (4 mg/kg) prior to laparotomy and complete exsanguination. Pancreata were carefully dissected from surrounding tissue and placed in cold (4°C) HBSS supplemented with 0.25% BSA (fraction V), 10 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 20 μg/ml gentamycin until processing (within an hour after tissue collection). Pancreatic tissue was finely minced into 1–2 mm^3 fragments in supplemented HBSS (without BSA) containing Liberase PI (Roche, Indianapolis, IN, USA) (0.75 mg/ml). The tissue was digested with agitation for 16–18 min in a shaking water bath at 37°C, and the reaction stopped by the addition of excess cold supplemented HBSS (containing BSA). The suspension was filtered through 500-μM nylon mesh, and washed four times in supplemented HBSS by centrifugation at 400 rpm, for 2 min without breaking. The final islet pellet was resuspended in HAM's-F12 medium supplemented with 1.2 mg/ml nicotinamide, 11 μg/ml isobutylmethyl xanthine, 0.5% BSA, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, and cultured at 37°C, 5% CO_2 in 100mm petri dishes. The culture medium was changed the day after isolation, and every second day thereafter.

Islet Dispersion

NPICC were collected and washed by gentle centrifugation at 400 rpm, for 2 min (no break) and the supernatant discarded. The NPICC pellet was washed twice in warm (37°C) dispersion medium (HBSS containing 1 mM EDTA, 10 mM HEPES, and 0.5% BSA), with gentle centrifugation as previously described. Supernatants after each wash were collected and combined. Following the final wash, the NPICC pellet was resuspended in warm dispersion medium incubated with gentle agitation at 37°C for 7 min. NPICC were then dispersed by the

addition of trypsin (25 μg/ml) and DNase (4 μg/ml) and incubated for 10 min at 37°C with gentle agitation. The dispersed cells were collected and combined with the previously collected supernatant fractions. Samples were centrifuged at 1200 rpm for 5 min and washed once in complete growth medium. The final pellet was resuspended in complete growth medium and samples counted by trypan blue exclusion.

Caspase Assay

The caspase assay was conducted using the Apo-1 fluorescent caspase-3,-7 assay (Promega) according to the manufacturer's directions. Sample loading was standardized either by the use of dispersed single islet cells (30,000 or 50,000) or by the use of the Cell-titre blue assay (Promega) on whole islets, prior to the addition of the Apo-1 reagent, by incubation for 1.5 h and assessment of fluorescence ($560_{EX}/590_{EM}$). Cells or whole islets from each sample were dispensed into wells of a 96-well tissue plate in duplicate. Samples were incubated with the substrate Z-DEVD-rhodamine 110 for 18 h prior to the determination of caspase-3,-7 activity by measurement of fluorescence intensity at 535 nm.

Assessment of Subdiploid DNA Content by Propidium Iodide (PI) Staining (PI Assay)

Aliquots of single cells from each islet treatment group were collected and washed twice in PBS. Final pellets were resuspended in PBS, to which cold absolute ethanol was added dropwise, to achieve a final concentration of 70%. Cells were stored at −20°C overnight prior to PI staining. Prior to staining, cells were washed twice in PBS, and the final pellet resuspended in 500 μl PI stock solution (50 μg/ml), to which 6.25 μl Nonidet P40 (10% stock solution in H_2O) and 8 μl RNaseA (1 mg/ml) was added. Samples were vortexed and incubated at room temperature, protected from light, for 30 min. Samples were immediately assessed by flow cytometry.

Western Blot for HO-1 and PARP-1 Proteins

HO-1 protein expression was determined in aliquots of 50,000 single (dispersed) islet cells. Cells were dispensed into Eppendorf tubes, centrifuged, and the supernatant discarded. Pellets were resuspended in 2× SDS-PAGE loading buffer and stored at −20°C until blot performed. After boiling for 5 min, proteins present in the islet cell lysates were separated in 10% SDS-PAGE gels and transferred to PVDF nylon membranes (Amersham, UK). Following blocking for 1 h in PBS + 5% nonfat milk, membranes were probed with antibodies specific for HO-1 (Stressgen (Victoria, Canada), rabbit polyclonal IgG, diluted 1:5000), and actin (Santa-Cruz (CA, USA) (C-2) mouse monoclonal IgG, diluted 1:100) for 1 h at room temperature with agitation. Primary antibodies were detected by binding to peroxidase-conjugated anti-rabbit and anti-mouse IgG (Calbiochem, CA, USA) (diluted 1:2000 in blocking solution, incubated for 1 h at room temperature with agitation), and revealed using a chemiluminescent detection system (Amersham, UK).

For the analysis of PARP-1 protein expression, equivalent numbers of whole islets were resuspended in 200 μl lysis buffer (62.5 mM Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, 5% β-mercaptoethanol) and sonicated for 20 s at 40% output. Lysates were heated to 65°C, for 15 min, and stored at −20°C prior to Western blotting. One hundred microliters of each sample was concentrated by centrifugation (Vivaspin 500 columns, Vivascience, Stonehouse, UK), and the resulting protein solution separated via electrophoresis in 8% SDS-PAGE gels. Proteins were transferred to PVDF nylon membranes, blocked in PBS + 5% nonfat milk + 0.1% Tween-20 for 1 h, and probed with antibodies to PARP-1 [mouse monoclonal C2-10 (Alexis Biochemicals, Lausen, Switzerland), diluted 1:500, overnight at 4°C with agitation] and actin (as above, 1 h at room temperature). Primary antibodies were detected with an anti-mouse-HRP secondary antibody (as above), and signals detected by chemiluminesence. HL-60 control samples were purchased from BioMol (Exeter, UK).

Statistical Analysis

Results represent mean values ± SEM of duplicate or triplicate samples. Statistical comparisons were performed by Student's *t*-test and considered significantly different when $p \leq 0.05$.

RESULTS

NPICC Are Highly Sensitive to Apoptosis Induction by Exposure to TNF-α and Cycloheximide in Combination

Islets from different species demonstrate different sensitivities to apoptotic stimuli (19). Porcine islets appear to be particularly sensitive to death stimuli, with adult porcine islets reportedly difficult to maintain for extended periods in culture (12,41). A dose–response study was performed to determine the apoptotic response of NPICC following 18-h stimulation with varying combinations of TNF-α and CHX and dispersed islets were assessed by caspase and PI assays. The results are shown in Figure 1.

TNF-α alone or in combination with CHX resulted in caspase-3,-7 enzyme activation ($p < 0.05$ at TNF-α concentrations ≥ 25 ng/ml) (Fig. 1A). The response to apoptotic stimulation was dependent on islet age and quality.

In contrast, the PI assay demonstrated a dose-dependent relationship between subdiploid DNA content of

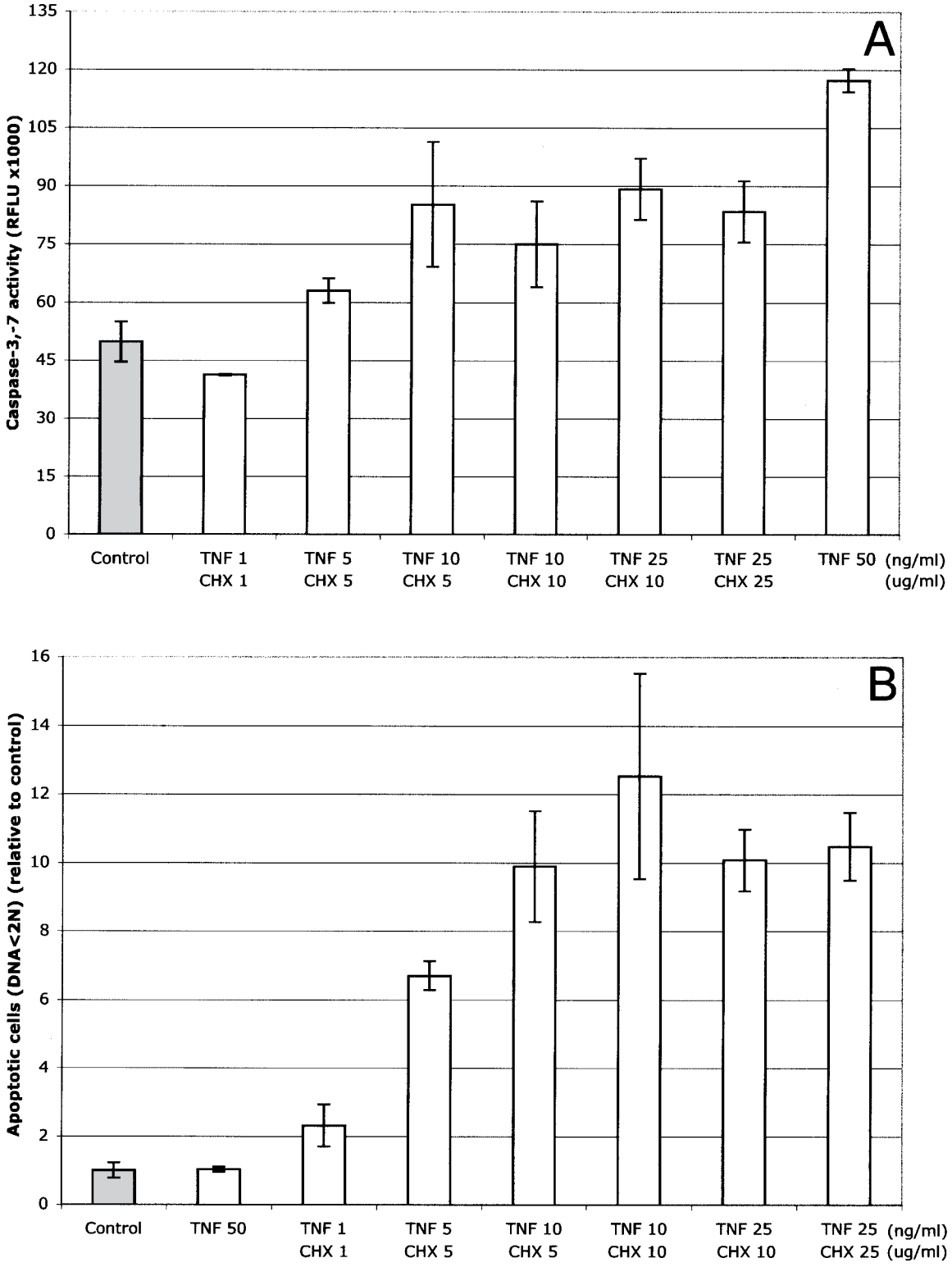

Figure 1. Apoptosis in NPICC following stimulation with TNF-α and CHX in combination. NPICC were cultured for 18 h with increasing combinations of TNF-α (ng/ml) and CHX (μg/ml) or with 50 ng/ml TNF-α alone. Caspase-3,-7 activity was measured in samples of 50,000 cells in a fluorimetric assay (A). Following dispersion into single cell suspensions, subdiploid DNA content was determined via PI staining and assessed by flow cytometry (B).

NPICC and the concentration of the apoptotic stimulus (Fig. 1B). TNF-α alone showed no detectable induction of apoptosis by the PI assay at this time point (18 h postexposure). However, given sufficient time, the subdiploid DNA analysis also demonstrates the presence of apoptosis, albeit at lower levels in comparison to TNF-α in combination with CHX (see below). In contrast, the lowest dose combination of TNF-α and CHX used (1 ng/ml and 1 μg/ml, respectively) was sufficient to induce apoptosis (2.3-fold higher than control). A 10-fold increase in the concentration of the apoptotic stimulus (10 ng/ml and 10 μg/ml, respectively) resulted in the attainment of a plateau level of apoptosis approximately 10-fold higher than control levels, which was not significantly altered following further concentration increases of the apoptotic stimulus.

Treatment of NPICC With Either Cobalt Protoporphyrin or Cobalt Mesoporphyrin Results in the Dose-Dependent Induction of Heme-Oxygenase 1

Treatment with either porphyrin resulted in dose-dependent upregulation of HO-1 expression. While as little as 30-min exposure to CoPP was sufficient to result in significant HO-1 expression (data not shown), porphyrins were maintained in the cultures for the duration of the experiments. Significant HO-1 expression was observed even in apoptotic conditions (Fig. 2). Control untreated NPICC (Fig. 2A) or NPICC treated with TNF-α/CHX only (Fig. 2B) showed little or no basal HO-1 protein expression. In the presence of TNF-α/CHX, low-level HO-1 expression was visible in NPICC treated with 10 μM CoPP. Significant increases in HO-1 expression were observed following treatment at concentrations equal or greater than 25 μM for both CoPP and CoMP. However, CoPP (Fig. 2A) stimulated HO-1 expression to a greater degree than CoMP (Fig. 2B).

HO-1 Induction Is Not Associated With Protection of NPICC From Apoptosis Induced by Combined Treatment With TNF-α and CHX

Treatment with 200 μM CoPP, in the absence of apoptotic stimulation, had no gross adverse effects on the islets, as judged by morphology (Fig. 3A and B). In NPICC cultured in the presence or absence of CoPP, the clusters were tightly packed, with clear inclusion membranes surrounding their external perimeter. As observed both in caspase (Fig. 3E) and PI (Fig. 3F) assays, islets cultured in the presence of porphyrin alone showed levels of apoptosis not significantly different from untreated control islets (CoPP $p = 0.422$ and 0.597 for caspase and PI assays, respectively).

While islets treated with porphyrins expressed HO-1 and did not appear to be adversely affected by the presence of the porphyrins in the cultures, islets treated with porphyrins, and subsequently exposed to TNF-α and CHX, still demonstrated susceptibility to apoptosis. The caspase assay (Fig. 3E) showed heightened levels of caspase-3,-7 activity in islets treated with apoptosis-inducing agents, regardless of the presence of porphyrins (50 μM CoPP and CoMP, $p < 0.035$). This result was reiter-

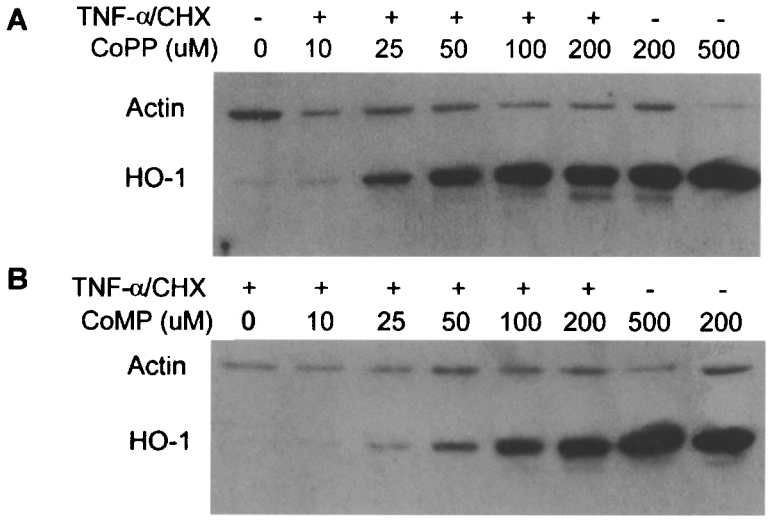

Figure 2. HO-1 expression in NPICC following treatment with increasing concentrations of CoPP (A) or CoMP (B). NPICC were cultured in the presence of 10–200 μM CoPP (A) or CoMP (B) for 24 h prior to stimulation with 25 ng/ml TNF-α in combination with 25 μg/ml CHX. Negative controls included untreated NPICC (A) and NPICC treated with TNF-α/CHX only (B). NPICC treated with 500 μM CoPP were used as a positive control.

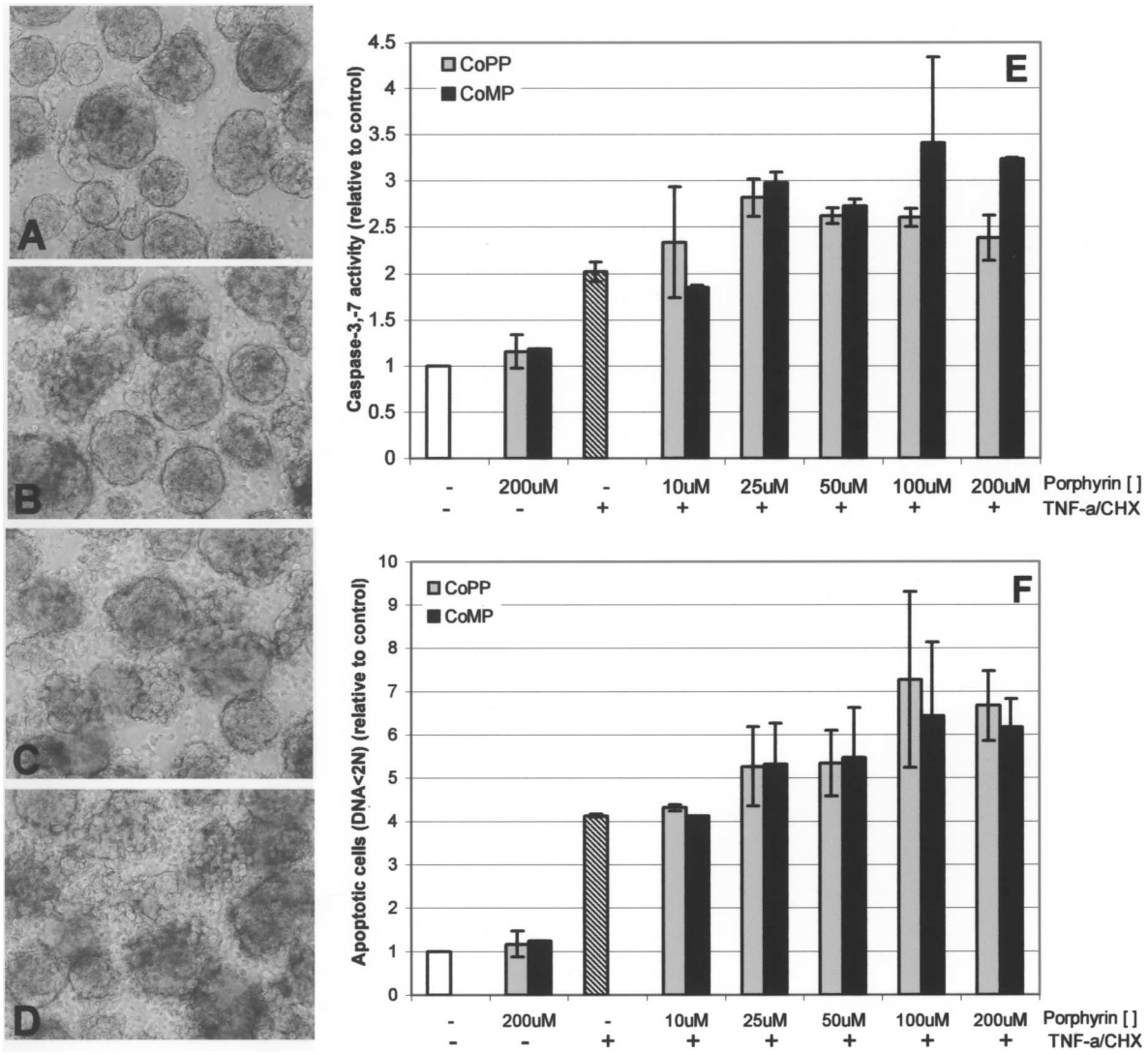

Figure 3. Effect of CoPP dose on NPICC morphology, caspase-3,-7 enzyme activity, and subdiploid DNA content in NPICC treated with TNF-α/CHX. (A) NPICC in standard culture conditions. (B) NPICC cultured in the presence of 200 μM CoPP. (C) NPICC cultured in the presence of TNF-α (1 ng/ml) and CHX (1 μg/ml) for 18 h. (D) NPICC cultured for 24 h with 200 μM CoPP, prior to apoptotic stimulation with TNF-α (1 ng/ml) and CHX (1 μg/ml) for 18 h. Following culture with porphyrins for 24 h [CoPP (gray columns) or CoMP (black columns)], NPICC were stimulated with TNF-α (25 ng/ml) and CHX (25 μg/ml) for 18 h. Caspase-3,-7 activation (E) and subdiploid DNA content (F) were evaluated in dispersed islets. Controls included untreated NPICC (open columns) and NPICC treated with TNF-α/CHX only (hatched columns). Results are the mean of two independent experiments.

ated in the PI assay, demonstrating increased subdiploid DNA content in TNF-α/CHX-stimulated islets at all porphyrin concentrations assessed (Fig. 3F). Therefore, while porphyrins on their own (200 μM) do not alter baseline levels of NPICC apoptosis, treatment with porphyrins is not able to prevent the apoptotic process induced by TNF-α/CHX stimulation. Indeed, levels of DNA degradation observed in the PI assay and caspase activity tended to increase with increasing porphyrin concentrations to a plateau at around 25 μM, suggesting a possible combinatorial effect.

The inability of porphyrin treatment to reduce levels of apoptosis in treated NPICC was also confirmed morphologically. Following stimulation with TNF-α alone, either at 50 or 100 ng/ml, minor morphological changes were evident. Increases in the number of single cells in the culture were evident surrounding the islets (at either concentration). The islets darkened slightly, and cells

detaching from the islets could be observed at the islet perimeter, obscuring the inclusion membrane, suggesting loss of cluster aggregation (data not shown). These changes were equally apparent at either 50 or 100 ng/ml. Treatment with the combination of TNF-α and CHX resulted in more obvious morphological changes (Fig. 3C). All islets were clearly dark in appearance, shedding cells at the periphery. The number of single cells in the culture had increased in comparison to control islets. In the case of smaller islets, the inclusion membrane was no longer present. The presence of CoPP in this culture was not able to inhibit the development of these features of islet damage (Fig. 3D).

Following Mild Apoptosis Induction, CoPP Treatment Increases HO-1 Expression and Reduces Caspase-3,-7 Enzyme Activation, But Does Not Inhibit Cell Death

Considering the high levels of apoptosis obtained following exposure to the combination of TNF-α and CHX, and the capacity of TNF-α alone to result in considerable caspase activation (Fig. 1), the subsequent experiments were undertaken using TNF-α alone to stimulate apoptosis. TNF-α alone at either 50 or 100 ng/ml required 48–72 h to result in peak levels of caspase activity and DNA fragmentation, with both concentrations showing similar levels of induction, although generally more consistent at a concentration of 100 ng/ml (data not shown). The effects of CoPP treatment were therefore assessed following this stimulation regimen, with subsequent investigations utilizing TNF-α at 100 ng/ml. Levels of apoptosis in NPICC cultured in the presence or absence of 200 μM CoPP were assessed following stimulation with 100 ng/ml TNF-α, with caspase-3,-7 enzyme activity assessed at 48 h following TNF-α stimulation and subdiploid DNA content at 72 h.

The results of the caspase assay on the dispersed islet cell fraction are presented in Figure 4A. No significant difference in caspase-3,-7 activation was observed between untreated control islets and islets cultured in the presence of CoPP. In contrast, a clear and significant reduction in caspase-3,-7 activation was observed in CoPP-treated islets following 48-h exposure to 100 ng/ml TNF-α when compared to controls ($p = 0.0036$) (Fig. 4A).

Contrary to the results of the caspase assay, no reductions in the level of DNA degradation were demonstrated by the PI assay (Fig. 4B). Assessment of DNA degradation present in cultures at 72 h post-TNF-α treatment indicated that, notwithstanding the inhibition of caspase enzymes in these cultures, apoptotic/necrotic processes were ongoing. Indeed, some experiments indicated that cell death may have been augmented by the presence of CoPP (data not shown).

The results of these experiments suggested that NPICC death following TNF-α stimulation was independent of caspase inhibition following CoPP treatment. In order to explore this hypothesis, the specific caspase-3,-7 inhibitor Ac-DEVD-CHO was used to block caspases following TNF-α stimulation.

As expected, Ac-DEVD-CHO almost completely inhibited caspase-3,-7 enzyme activity in NPICC following 48-h stimulation with TNF-α (Fig. 5A). Significant reductions in caspase-3,-7 activity were observed following the administration of both concentrations of the inhibitor in comparison to control levels ($p < 0.0005$ for both concentrations). Caspase enzyme activity observed following culture in the presence of 200 μM CoPP was similar to that observed in untreated control NPICC cultures.

While exposure to Ac-DEVD-CHO in the absence of apoptotic stimulation did not result in significant alterations in the level of DNA degradation in comparison to control islets (data not shown), treatment with the caspase inhibitor was not able to reduce levels of DNA degradation (i.e., cell death) in NPICC treated with TNF-α (72 h) (Fig. 5B). In fact, islets treated with the inhibitor demonstrated higher levels of cell death in contrast to TNF-α alone. In confirmation of our previous observations, pretreatment of NPICC with CoPP resulted in responses resembling those following specific caspase-3,-7 inhibition. Such results suggest that CoPP in this model inhibits caspase-3,-7 enzyme activation, which is, however, either unrelated to, or insufficient to inhibit, NPICC death.

Lack of PARP-1 Cleavage Following Apoptotic Stimulation in the Presence of CoPP Demonstrates Loss of the Apoptotic Phenotype

Assessment of PARP-1 cleavage patterns was performed in order to assess apoptosis in the NPICC cultures. In apoptotic conditions, the full-length (116 kDa) active protein is cleaved, primarily by caspase-3, to yield an 89-kDa fragment, inactivating the enzyme and inhibiting DNA repair. Failure to cleave the activated enzyme results in sustained enzymatic activity, and the depletion of cellular energy pools, ultimately resulting in necrosis (37).

The cleavage patterns of PARP-1 following apoptosis induction by TNF-α in NPICC cultured for up to 96 h in the absence of CoPP showed the presence of the 89-kDa fragment, indicating the activation of caspase-mediated apoptosis (Fig. 6). Culture in the presence of CoPP alone did not activate PARP-1 cleavage. Furthermore, while at 48 h post-TNF-α treatment the 89-kDa fragment was present in cultures containing TNF-α and CoPP, at 72 and 96 h the 89-kDa fragment was no longer clearly visible. This result suggests a kinetic switch in the mechanism of islet death, with loss of the classic apoptotic phenotype occurring between 48 and

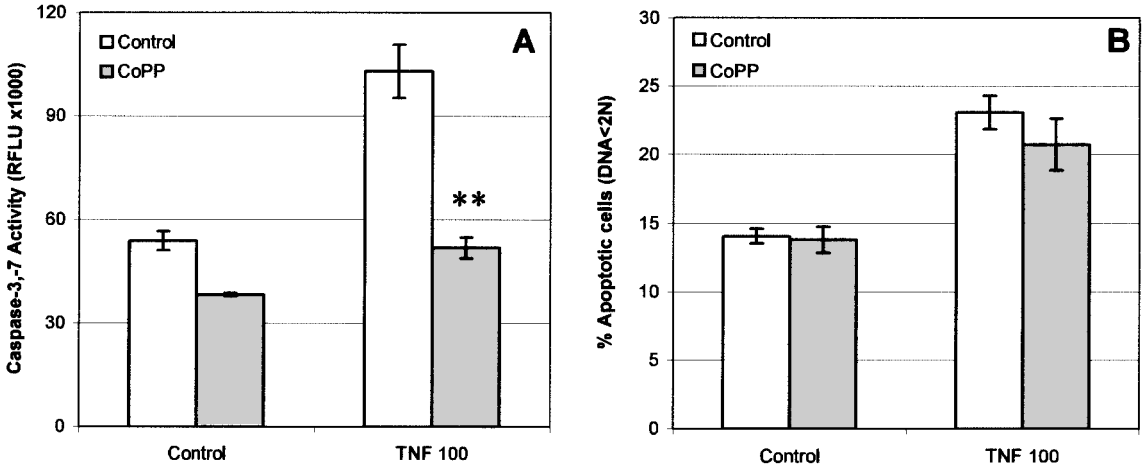

Figure 4. The effect of CoPP treatment on caspase-3,-7 activity and DNA fragmentation in NPICC treated with TNF-α only. NPICC were cultured for 24 h in the presence (gray columns) or absence (open columns) of 200 μM CoPP prior to stimulation with TNF-α alone at 100 ng/ml. (A) Caspase-3,-7 activity (48 h post-TNF-α exposure), and (B) subdiploid DNA content (72 h post-TNF-α exposure) were evaluated in dispersed NPICC cells. All samples were assessed in duplicate (**$p < 0.01$).

72 h post-TNF-α exposure. Necrotic NPICC (treated with 3 mM H_2O_2) did not demonstrate PARP-1 cleavage.

DISCUSSION

Apoptosis is a major cause of islet loss following isolation and transplantation, and strategies designed to reduce the process are necessary if long-term islet survival is to be achieved (20,44). The process of apoptosis is complex, initiated by a number of different stimuli such as stress, DNA damage, or cytokines, and can be executed by several different pathways (1,28).

Islets isolated from different species demonstrate different robustness to isolation, in vitro culture, and apoptotic stimulation (19). In addition, islets from immature (neonatal) animals vary greatly to those from the adult (3,33). Indeed, adult porcine islets are difficult and

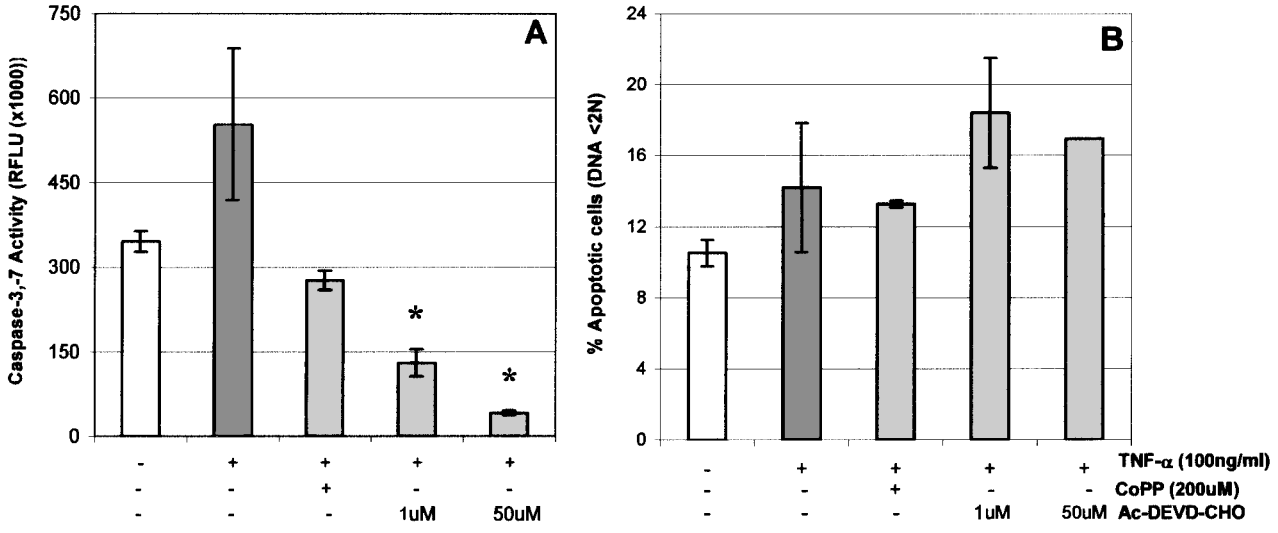

Figure 5. The effect of Ac-DEVD-CHO treatment on caspase-3,-7 enzyme activity (A) and subdiploid DNA content (B) following stimulation with TNF-α. After 48-h stimulation with 100 ng/ml TNF-α, caspase-3,-7 activity was assessed via fluorimetry in control and treated NPICC. Cell number was standardized using the Cell-titre blue assay (Promega). Samples were assessed in duplicate. At 72 h after TNF-α addition (100 ng/ml), NPICC were dispersed into single cell suspensions for the assessment of subdiploid DNA content in duplicate samples by flow cytometry. Examples are representative of two independent experiments. *$p < 0.0005$ in comparison to control.

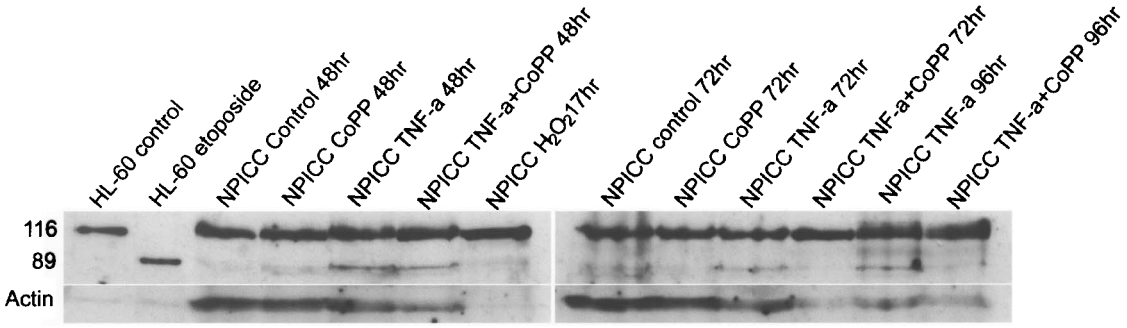

Figure 6. PARP-1 cleavage in NPICC following exposure to TNF-α in the presence or absence of CoPP. Following pretreatment for 24 h with CoPP (200 μM), NPICC were exposed to TNF-α for up to 96 h. At 24-h intervals, NPICC were collected and proteins extracted via sonication in PARP-1 lysis buffer. Following electrophoresis of protein lysates by SDS-PAGE (8% gel) and transfer to PVDF membranes, the membranes were probed with anti-PARP-1 (C2-10) and anti-actin antibodies. Signals were detected by chemiluminesence. NPICC treated with 3 mM H_2O_2 for 17 h were utilized as a control for necrosis.

costly to isolate, and are reknown for being particularly difficult to maintain in standard culture for extended periods (12,50), while neonatal porcine islets require a much simpler isolation methodology and can be maintained for at least several weeks in culture (30,34). For these reasons, NPICC were selected as the most suitable islets for these studies. The aim of this study was to assess the ability of CoPP to protect NPICC from death following stimulation with proapoptotic agents.

Several important observations were noted when standard apoptosis-inducing regimens were applied to these NPICC cultures. Firstly, in this study, a 10-fold increase in the levels of apoptosis was induced in NPICC following exposure to 25 ng/ml TNF-α and 25 μg/ml CHX. These concentrations were lower than those that induced only low levels (5–10%) of apoptosis in murine islets (TNF-α 5000 U/ml, CHX 50 μg/ml) (46). Secondly, even at concentrations of TNF-α/CHX that do not lead to the induction of apoptosis in murine islets (8,14), treatment of NPICC resulted in the induction of considerable levels of apoptosis. Assessment of caspase enzyme activation indicated that the low-dose combinations of TNF-α/CHX were also able to result in increases in caspase-3,-7 enzyme activity. Such observations underline the innate sensitivity of neonatal porcine islets to apoptosis, in contrast to rodent or human islets, which generally require exposure to multiple proapoptotic agents in order to activate the apoptotic process (11, 18,52).

Treatment of NPICC with either CoPP or CoMP, in the presence or absence of apoptotic stimulation, resulted in a dose-dependent upregulation of HO-1 protein as demonstrated by Western blot. CoPP appeared to induce a slightly higher level of HO-1 expression than CoMP, a result that has also been observed following porphyrin treatment of porcine aortic endothelial cells in our laboratory (unpublished observations). Treatment with 200 μM CoPP, which induced high levels of HO-1 protein expression, did not result in NPICC toxicity in terms of viability, morphology, and analyses of apoptosis.

Seemingly beneficial effects were noted following CoPP treatment of TNF-α-stimulated islets (i.e., the reduction in caspase-3,-7 enzyme activity). However, this was not associated with reduced levels of cell death in NPICC cultures. Similar observations following caspase inhibition have been reported in other studies (6,10). Indeed, in some cases the level of cell death subsequent to exposure to TNF-α/CHX seemed to be increased following the addition of CoPP. Treatment of NPICC with Ac-DEVD-CHO was also insufficient to inhibit DNA fragmentation, confirming that the inhibition of caspases-3,-7 is unable to alter the commitment to death following stimulation with TNF-α. The similarity of the response to CoPP with that observed following treatment with the caspase-3,-7-specific inhibitor Ac-DEVD-CHO suggests that the effect of CoPP in this instance could be a direct inhibitory effect on caspase enzymes. Indeed, inhibition of caspase activity has recently been described as a direct property of metal porphyrins, unrelated to HO-1 induction or activity (5). The results of this study therefore appear to be more related to the direct effect of CoPP on caspase activity than its HO-1-inducing effect.

At least two possibilities may explain the observation of ongoing cell death despite the inhibition of caspase activity. The first is that TNF-α can cause islet apoptosis via the activation of a caspase-independent pathway, or secondly, that by inhibiting TNF-α-mediated caspase activation, an alternative cell death mechanism (possibly necrosis) is activated. Evidence from this study and from others (2) suggest that the second scenario is the most

likely in this model. Therefore, the failure of caspase inhibition to protect cells from death may be related to the induction of a switch in death programming towards necrosis (35,37).

The processes of apoptosis and necrosis are interrelated and most likely represent two extreme ends of a complex spectrum of death mechanisms (13,40). The reported switch from apoptosis to necrosis following caspase inhibition is believed to be due in large part to the regulation of the DNA repair enzyme PARP-1. Indeed, following DNA damage, PARP-1 is activated to take part in the DNA repair mechanism. The activated enzyme caspase-3 cleaves PARP-1 to inactivate it, inhibiting the DNA repair process. In the absence of caspase-3 activity, PARP-1 remains functional, and in the continued presence of a damaging stimulus, results in the depletion of cellular energy pools, and the induction of cellular necrosis (26).

In this study, incomplete PARP-1 cleavage to the 89-kDa fragment was observed following stimulation with TNF-α, indicating activation of the caspase-mediated apoptotic pathway. In the presence of CoPP, the cleavage fragment is present at the 48-h time point. However, at 72 and 96 h post-TNF-α exposure, cleavage was no longer observed, notwithstanding high levels of DNA fragmentation at these time points. This suggests loss of the classic apoptotic phenotype and a possible switch towards necrosis. Indeed, NPICC induced to undergo necrosis by exposure to H_2O_2 also lacked expression of the 89-kDa fragment.

Alternatively, PARP-1 hyperactivity can also result in caspase-independent, apoptosis-inducing factor (AIF)-mediated apoptosis (17,58). Indeed, further analyses will be required to fully rule out such a possibility. In addition, the very nature of the islet mass (i.e., an aggregation of various cell types of endocrine and nonendocrine origin) may create complexity in the interpretation of these data. However, results obtained in other similar studies (2), and the observed effects of CoPP in other cell models in this laboratory (unpublished data), would suggest that the net effect of caspase inhibition by CoPP in this study is indeed a switch towards necrosis.

The results of this study suggest that the use of CoPP as a cytoprotective agent in vitro appears to be of little benefit, or possibly even detrimental, in this model. This result was not altogether expected in the light of previous studies published by Pileggi and colleagues (46,47), in which protective effects were demonstrated following treatment of murine islets with FePP in vitro, and CoPP in vivo. Several possible explanations for these discrepancies may be proposed.

In the first instance, we utilized a different metalloporphyrin. Indeed, CoPP was preferred to FePP for these in vitro studies, as CoPP has been reported to be the most efficacious metalloporphyrin inducer of HO-1, an important antiapoptotic and cytoprotective protein (53). Furthermore, rodent islets are more robust and resilient to damage than the porcine islets used in this study. Indeed, the same apoptotic stimulus applied in the Pileggi et al. study (TNF-α/CHX) (46) resulted in levels of apoptosis greater than 40% in this model, while levels of less than 10% were reported in the murine study. In addition, it is possible that the beneficial effects of CoPP observed on transplanted murine islets in the in vivo situation are mostly due to alterations of the recipient microenvironment. In such a situation, in vivo administration of CoPP, resulting in upregulation of HO-1 expression and immunosuppressive effects, may therefore create an anti-inflammatory environment into which transplanted islets would be subject to a greatly reduced posttransplant burden. In this context, in the presence of reduced (or absent) apoptotic stimulation, the actions of CoPP could be expected to enhance islet engraftment.

In summary, NPICC are intrinsically sensitive to cytokine (TNF-α)-mediated death in vitro. Exposure of NPICC to the cytoprotective agent CoPP is nontoxic and elicits strong induction of the antiapoptotic protein HO-1 in vitro. However, the use of CoPP appears unable to prevent cell death following apoptotic stimulation in vitro. Indeed, caspase-3,-7 inhibition by the compound results in an alteration of the death phenotype. Together, these data suggest that as far as NPICC are concerned, CoPP should not be viewed as an NPICC protective agent in vitro, although its strong HO-1-inducing capacity may still benefit NPICC survival in vivo.

ACKNOWLEDGMENTS: This work was supported by a MIUR (Ministero dell'Università e della Ricerca) research grant (Italy), protocol # 2004067425_006, CORIT (Consortium for Research in Organ Transplantation), and the EU FP6 Integrated Project "Xenome," contract # LSHB-CT-2006-037377.

REFERENCES

1. Abdelli, S.; Ansite, J.; Roduit, R.; Borsello, T.; Matsumoto, I.; Sawada, T.; Allaman-Pillet, N.; Henry, H.; Beckmann, J. S.; Hering, B. J.; Bonny, C. Intracellular stress signaling pathways activated during human islet preparation and following acute cytokine exposure. Diabetes 53: 2815–2823; 2004.
2. Aikin, R.; Rosenberg, L.; Paraskevas, S.; Maysinger, D. Inhibition of caspase-mediated PARP-1 cleavage results in increased necrosis in isolated islets of Langerhans. J. Mol. Med. 82:389–397; 2004.
3. Asplund, K.; Westman, S.; Hellerstrom, C. Glucose stimulation of insulin secretion from the isolated pancreas of foetal and newborn rats. Diabetologia 5:260–262; 1969.
4. Bennet, W.; Sundberg, B.; Groth, C. G.; Brendel, M. D.; Brandhorst, D.; Brandhorst, H.; Bretzel, R. G.; Elgue, G.; Larsson, R.; Nilsson, B.; Korsgren, O. Incompatibility between human blood and isolated islets of Langerhans: A finding with implications for clinical intraportal islet transplantation? Diabetes 48:1907–1914; 1999.

5. Blumenthal, S. B.; Kiemer, A. K.; Tiegs, G.; Seyfried, S.; Holtje, M.; Brandt, B.; Holtje, H. D.; Zahler, S.; Vollmar, A. M. Metalloporphyrins inactivate caspase-3 and -8. FASEB J. 19:1272–1279; 2005.
6. Brandhorst, D.; Kumarasamy, V.; Maatoui, A.; Alt, A.; Bretzel, R. G.; Brandhorst, H. Porcine islet graft function is affected by pretreatment with a caspase-3 inhibitor. Cell Transplant. 15:311–317; 2006.
7. Buhler, L.; Deng, S.; O'Neil, J.; Kitamura, H.; Koulmanda, M.; Baldi, A.; Rahier, J.; Alwayn, I. P.; Appel, J. Z.; Awwad, M.; Sachs, D. H.; Weir, G.; Squifflet, J. P.; Cooper, D. K.; Morel, P. Adult porcine islet transplantation in baboons treated with conventional immunosuppression or a non-myeloablative regimen and CD154 blockade. Xenotransplantation 9:3–13; 2002.
8. Campbell, I. L.; Iscaro, A.; Harrison, L. C. IFN-gamma and tumor necrosis factor-alpha. Cytotoxicity to murine islets of Langerhans. J. Immunol. 141:2325–2329; 1988.
9. Cardona, K.; Korbutt, G. S.; Milas, Z.; Lyon, J.; Cano, J.; Jiang, W.; Bello-Laborn, H.; Hacquoil, B.; Strobert, E.; Gangappa, S.; Weber, C. J.; Pearson, T. C.; Rajotte, R. V.; Larsen, C. P. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nat. Med. 12:304–306; 2006.
10. Carter, B. Z.; Kornblau, S. M.; Tsao, T.; Wang, R. Y.; Schober, W. D.; Milella, M.; Sung, H. G.; Reed, J. C.; Andreeff, M. Caspase-independent cell death in AML: caspase inhibition in vitro with pan-caspase inhibitors or in vivo by XIAP or Survivin does not affect cell survival or prognosis. Blood 102:4179–4186; 2003.
11. Cattan, P.; Berney, T.; Schena, S.; Molano, R. D.; Pileggi, A.; Vizzardelli, C.; Ricordi, C.; Inverardi, L. Early assessment of apoptosis in isolated islets of Langerhans. Transplantation 71:857–862; 2001.
12. Cavanagh, T. J.; Lakey, J. R.; Dwulet, F.; Wright, M. J.; Wile, K.; Albertson, T.; Fetterhoff, T. Improved pig islet yield and post-culture recovery using Liberase PI purified enzyme blend. Transplant. Proc. 30:367; 1998.
13. Chiarugi, A. "Simple but not simpler": Toward a unified picture of energy requirements in cell death. FASEB J. 19:1783–1788; 2005.
14. Chong, M. M.; Thomas, H. E.; Kay, T. W. Suppressor of cytokine signaling-1 regulates the sensitivity of pancreatic beta cells to tumor necrosis factor. J. Biol. Chem. 277: 27945–27952; 2002.
15. Close, N.; Anand, R.; Hering, B.; Eggerman, T. NIH-Supported National Islet Transplantation Registry. Cell Biochem. Biophys. 40:9–18; 2004.
16. Contreras, J. L.; Bilbao, G.; Smyth, C.; Eckhoff, D. E.; Xiang, X. L.; Jenkins, S.; Cartner, S.; Curiel, D. T.; Thomas, F. T.; Thomas, J. M. Gene transfer of the Bcl-2 gene confers cytoprotection to isolated adult porcine pancreatic islets exposed to xenoreactive antibodies and complement. Surgery 130:166–174; 2001.
17. Cregan, S. P.; Dawson, V. L.; Slack, R. S. Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene 23:2785–2796; 2004.
18. Delaney, C. A.; Pavlovic, D.; Hoorens, A.; Pipeleers, D. G.; Eizirik, D. L. Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. Endocrinology 138:2610–2614; 1997.
19. Eizirik, D. L.; Pipeleers, D. G.; Ling, Z.; Welsh, N.; Hellerstrom, C.; Andersson, A. Major species differences between humans and rodents in the susceptibility to pancreatic beta-cell injury. Proc. Natl. Acad. Sci. USA 91: 9253–9256; 1994.
20. Emamaullee, J. A.; Shapiro, A. M. Interventional strategies to prevent beta-cell apoptosis in islet transplantation. Diabetes 55:1907–1914; 2006.
21. Fenjves, E. S.; Ochoa, M. S.; Gay-Rabinstein, C.; Molano, R. D.; Pileggi, A.; Mendez, A. J.; Inverardi, L.; Ricordi, C. Adenoviral gene transfer of erythropoietin confers cytoprotection to isolated pancreatic islets. Transplantation 77:13–18; 2004.
22. Fulda, S.; Meyer, E.; Debatin, K. M. Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. Oncogene 21: 2283–2294; 2002.
23. Glassford, A.; Lee, J. E.; Xu, L.; Giffard, R. G. Caspase inhibitors reduce the apoptotic but not necrotic component of kainate injury in primary murine cortical neuronal cultures. Neurol. Res. 24:796–800; 2002.
24. Grey, S. T.; Arvelo, M. B.; Hasenkamp, W.; Bach, F. H.; Ferran, C. A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets. J. Exp. Med. 190:1135–1146; 1999.
25. Gunther, L.; Berberat, P. O.; Haga, M.; Brouard, S.; Smith, R. N.; Soares, M. P.; Bach, F. H.; Tobiasch, E. Carbon monoxide protects pancreatic beta-cells from apoptosis and improves islet function/survival after transplantation. Diabetes 51:994–999; 2002.
26. Ha, H. C.; Snyder, S. H. Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc. Natl. Acad. Sci. USA 96:13978–13982; 1999.
27. Hering, B. J.; Wijkstrom, M.; Graham, M. L.; Hardstedt, M.; Aasheim, T. C.; Jie, T.; Ansite, J. D.; Nakano, M.; Cheng, J.; Li, W.; Moran, K.; Christians, U.; Finnegan, C.; Mills, C. D.; Sutherland, D. E.; Bansal-Pakala, P.; Murtaugh, M. P.; Kirchhof, N.; Schuurman, H. J. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nat. Med. 12:301–303; 2006.
28. Hui, H.; Dotta, F.; Di Mario, U.; Perfetti, R. Role of caspases in the regulation of apoptotic pancreatic islet beta-cells death. J. Cell. Physiol. 200:177–200; 2004.
29. Hui, H.; Nourparvar, A.; Zhao, X.; Perfetti, R. Glucagon-like peptide-1 inhibits apoptosis of insulin-secreting cells via a cyclic 5′-adenosine monophosphate-dependent protein kinase A- and a phosphatidylinositol 3-kinase-dependent pathway. Endocrinology 144:1444–1455; 2003.
30. Juang, J. H.; Hsu, B. R.; Kuo, C. H.; Fu, S. H.; Lee, W. C.; Yao, N. K. Neonatal porcine pancreas as a source of islet transplantation. Transplant. Proc. 33:757–758; 2001.
31. Kirchhof, N.; Shibata, S.; Wijkstrom, M.; Kulick, D. M.; Salerno, C. T.; Clemmings, S. M.; Heremans, Y.; Galili, U.; Sutherland, D. E.; Dalmasso, A. P.; Hering, B. J. Reversal of diabetes in non-immunosuppressed rhesus macaques by intraportal porcine islet xenografts precedes acute cellular rejection. Xenotransplantation 11:396–407; 2004.
32. Komoda, H.; Miyagawa, S.; Kubo, T.; Kitano, E.; Kitamura, H.; Omori, T.; Ito, T.; Matsuda, H.; Shirakura, R. A study of the xenoantigenicity of adult pig islets cells. Xenotransplantation 11:237–246; 2004.
33. Korbutt, G. S.; Aspeslet, L.; Ao, Z.; Warnock, G. L.; Ezekowitz, J.; Koshal, A.; Rajotte, R. V.; Yatscoff, R. W. Porcine islet cell antigens are recognized by xenoreactive natural human antibodies of both IgG and IgM subtypes. Transplant. Proc. 28:837–838; 1996.

34. Korbutt, G. S.; Elliott, J. F.; Ao, Z.; Smith, D. K.; Warnock, G. L.; Rajotte, R. V. Large scale isolation, growth, and function of porcine neonatal islet cells. J. Clin. Invest. 97: 2119–2129; 1996.
35. Lemaire, C.; Andreau, K.; Souvannavong, V.; Adam, A. Inhibition of caspase activity induces a switch from apoptosis to necrosis. FEBS Lett. 425:266–270; 1998.
36. Liu, Z. M.; Chen, G. G.; Ng, E. K.; Leung, W. K.; Sung, J. J.; Chung, S. C. Upregulation of heme oxygenase-1 and p21 confers resistance to apoptosis in human gastric cancer cells. Oncogene 23:503–513; 2004.
37. Los, M.; Mozoluk, M.; Ferrari, D.; Stepczynska, A.; Stroh, C.; Renz, A.; Herceg, Z.; Wang, Z. Q.; Schulze-Osthoff, K. Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling. Mol. Biol. Cell 13: 978–988; 2002.
38. MacKenzie, D. A.; Hullett, D. A.; Sollinger, H. W. Xenogeneic transplantation of porcine islets: An overview. Transplantation 76:887–891; 2003.
39. Nanji, S. A.; Shapiro, A. M. Advances in pancreatic islet transplantation in humans. Diabetes Obes. Metab. 8:15–25; 2006.
40. Nicotera, P.; Melino, G. Regulation of the apoptosis-necrosis switch. Oncogene 23:2757–2765; 2004.
41. Nielsen, T. B.; Yderstraede, K. B.; Rasmussen, H. J.; Beck-Nielsen, H. Isolation of neonatal porcine islet tissue and transplantation into diabetic mice. A methodological evaluation. Scand. J. Lab. Anim. Sci. 27:129–137; 2000.
42. Onkamo, P.; Vaananen, S.; Karvonen, M.; Tuomilehto, J. Worldwide increase in incidence of Type I diabetes—the analysis of the data on published incidence trends. Diabetologia 42:1395–1403; 1999.
43. Otterbein, L. E.; Soares, M. P.; Yamashita, K.; Bach, F. H. Heme oxygenase-1: Unleashing the protective properties of heme. Trends Immunol. 24:449–455; 2003.
44. Paraskevas, S.; Maysinger, D.; Wang, R.; Duguid, T. P.; Rosenberg, L. Cell loss in isolated human islets occurs by apoptosis. Pancreas 20:270–276; 2000.
45. Pileggi, A.; Cattan, P.; Berney, T.; Molano, R. D.; Vizzardelli, C.; Ricordi, C.; Bach, F. H.; Inverardi, L. HO-1 upregulation protects the pancreatic cell line betaTC3 from cytokines and Fas-induced apoptosis. Transplant. Proc. 33:266–267; 2001.
46. Pileggi, A.; Molano, R. D.; Berney, T.; Cattan, P.; Vizzardelli, C.; Oliver, R.; Fraker, C.; Ricordi, C.; Pastori, R. L.; Bach, F. H.; Inverardi, L. Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved in vivo function after transplantation. Diabetes 50: 1983–1991; 2001.
47. Pileggi, A.; Molano, R. D.; Berney, T.; Ichii, H.; San Jose, S.; Zahr, E.; Poggioli, R.; Linetsky, E.; Ricordi, C.; Inverardi, L. Prolonged allogeneic islet graft survival by protoporphyrins. Cell Transplant. 14:85–96; 2005.
48. Riachy, R.; Vandewalle, B.; Kerr Conte, J.; Moerman, E.; Sacchetti, P.; Lukowiak, B.; Gmyr, V.; Bouckenooghe, T.; Dubois, M.; Pattou, F. 1,25-dihydroxyvitamin D3 protects RINm5F and human islet cells against cytokine-induced apoptosis: implication of the antiapoptotic protein A20. Endocrinology 143:4809–4819; 2002.
49. Ribeiro, M. M.; Klein, D.; Pileggi, A.; Molano, R. D.; Fraker, C.; Ricordi, C.; Inverardi, L.; Pastori, R. L. Heme oxygenase-1 fused to a TAT peptide transduces and protects pancreatic beta-cells. Biochem. Biophys. Res. Commun. 305:876–881; 2003.
50. Ricordi, C.; Socci, C.; Davalli, A. M.; Staudacher, C.; Baro, P.; Vertova, A.; Sassi, I.; Gavazzi, F.; Pozza, G.; Di Carlo, V. Isolation of the elusive pig islet. Surgery 107: 688–694; 1990.
51. Ricordi, C.; Strom, T. B. Clinical islet transplantation: Advances and immunological challenges. Nat. Rev. Immunol. 4:259–268; 2004.
52. Saldeen, J. Cytokines induce both necrosis and apoptosis via a common Bcl-2-inhibitable pathway in rat insulin-producing cells. Endocrinology 141:2003–2010; 2000.
53. Shan, Y.; Pepe, J.; Lu, T. H.; Elbirt, K. K.; Lambrecht, R. W.; Bonkovsky, H. L. Induction of the heme oxygenase-1 gene by metalloporphyrins. Arch. Biochem. Biophys. 380:219–227; 2000.
54. Shapiro, A. M.; Nanji, S. A.; Lakey, J. R. Clinical islet transplant: current and future directions towards tolerance. Immunol. Rev. 196:219–236; 2003.
55. Urusova, I. A.; Farilla, L.; Hui, H.; D'Amico, E.; Perfetti, R. GLP-1 inhibition of pancreatic islet cell apoptosis. Trends Endocrinol. Metab. 15:27–33; 2004.
56. Wagner, M.; Cadetg, P.; Ruf, R.; Mazzucchelli, L.; Ferrari, P.; Redaelli, C. A. Heme oxygenase-1 attenuates ischemia/reperfusion-induced apoptosis and improves survival in rat renal allografts. Kidney Int. 63:1564–1573; 2003.
57. Ye, J.; Laychock, S. G. A protective role for heme oxygenase expression in pancreatic islets exposed to interleukin-1beta. Endocrinology 139:4155–4163; 1998.
58. Yu, S. W.; Wang, H.; Poitras, M. F.; Coombs, C.; Bowers, W. J.; Federoff, H. J.; Poirier, G. G.; Dawson, T. M.; Dawson, V. L. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259–263; 2002.
59. Zhang, N.; Richter, A.; Suriawinata, J.; Harbaran, S.; Altomonte, J.; Cong, L.; Zhang, H.; Song, K.; Meseck, M.; Bromberg, J.; Dong, H. Elevated vascular endothelial growth factor production in islets improves islet graft vascularization. Diabetes 53:963–970; 2004.